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to the memory of my father

ABSTRACT

Bacteria have various ways to sense environmental signals and to adapt their behavior and physiology through different signaling systems. Secondary messenger signaling, amplified by enzymatic activity, rapidly transmits a signal in the cell resulting in allosteric functional control. Cyclic diguanosine monophosphate (c-di-GMP) is a novel global secondary messenger, that is found exclusively in bacteria and is involved in fundamental bacterial behavior such as motility, sessility and virulence. Regulation of virulence by c-di-GMP signaling is crucial for many pathogens.

The aim of this thesis was to study the potential role of c-di-GMP in bacterial-host interactions using *Salmonella enterica* serovar Typhimurium as a model system. We wanted to study the effect of c-di-GMP on virulence phenotypes and to identify the components and mechanisms through which c-di-GMP mediates its effects.

Using the colon carcinoma cell line HT-29 we found that high levels of intracellular c-di-GMP inhibited invasion of *S. typhimurium* into epithelial cells, and induction, by *S. typhimurium* of production of the proinflammatory cytokine itnerleukine-8 (IL-8) from epithelial cells. This suggests that c-di-GMP negatively regulates acute virulence phenotypes of *S. typhimurium*. Inhibition of virulence phenotypes is partially mediated through biofilm components; the exopolysaccharides cellulose and capsule, as well as the biofilm regulator CsgD. C-di-GMP also interferes with the secretion of SopE2, a *S. typhimurium* effector protein, as well as of flagellin, both of which are secreted by Type Three Secretion Systems.

GGDEF and EAL domain proteins are di-guanylate cyclases and phosphodiesterases that synthesize and degrade c-di-GMP, respectively. These proteins amplify the primary signal through a local or global change in the c-di-GMP concentration, and their specific activity determines the phenotypic output. We did a comprehensive study of *S. typhimurium* mutants of GGDEF/EAL domain proteins that revealed distinct groups of proteins that are involved in invasion, IL-8 production and colonization in streptomycin-treated mice. The distinct groups of proteins suggest non-redundancy and specific, localized activity of the secondary messenger towards regulatory targets.

C-di-GMP is involved in the regulation of biofilm formation. However, the role of biofilm formation in bacterial-host interaction of commensal *Escherichia coli* has not been studied in detail. So, we investigated the effect of the extracellular matrix components cellulose and curli fimbriae to bacterial adherence, internalization and induction

of the pro-inflammatory cytokine IL-8 in HT-29 cells. Cellulose and curli had differential effects; while curli fimbriae promoted adherence, internalization and IL-8 production, cellulose expression in the curli-expressing background inhibited these phenotypes. Curli-bound flagellin was highly immunostimulatory. In addition, our studies revealed two highly immunostimulatory flagellin sequences from commensal *E. coli* isolates. These flagellin sequences belong to the EC2 group of *E. coli* flagellins, which are closely related to *S. typhimurium* FliC flagellin, presumably already present in a common ancestor of *E. coli* and *S. typhimurium*.

LIST OF PUBLICATIONS

This thesis is based on the following papers, which are referred to in the text by their roman numerals:

I **Lamprokostopoulou A**, Monteiro C, Rhen M, Römling U. Cyclic di-GMP signalling controls virulence properties of *Salmonella enterica* serovar Typhimurium at the mucosal lining. Environ Microbiol. 2010. 12(1):40-53.

II **Lamprokostopoulou A**, Ahmad I, Streck E, Hardt WD, Römling U. Contribution of GGDEF-EAL domain proteins to *Salmonella typhimurium* virulence phenotypes.*Manuscript*

III Wang X, Rochon M, **Lamprokostopoulou A**, Lünsdorf H, Nimtz M, Römling U. Impact of biofilm matrix components on interaction of commensal *Escherichia coli* with the gastrointestinal cell line HT-29. Cell Mol Life Sci. 2006. 63(19-20):2352-63.

IV Ramos NL, **Lamprokostopoulou A**, Chapman TA, Chin JC, Römling U, Brauner A, Katouli M. Characteristics of translocating *Escherichia coli* and the interleukin-8 response to infection. *Manuscript*

Supplement Römling U, Jonas K, Melefors Ö, Grantcharova N, **Lamprokostopoulou A**. Hierarchical control of rdar morphotype development of *Salmonella enterica*. *In* The Second Messenger Cyclic Diguanylate, ASM press. *Review*

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LIST OF ABBREVIATIONS

GI	Gastrointestinal
PPs	Peyers patches
IELs	Intraepithelial lymphocytes
PAMP	Pathogen-associated molecular patterns
GALT	Gut-associated lymphoid tissue
MLN	Mesenteric lymph nodes
Ag	Antigen
IgA	Immunoglobbulin A
M	Microfold
PRRs	Pattern recognition receptors
LPS	Lipopolysaccharide
TLRs	Toll-like receptors
Ipaf	Interleukin-converting protease-activating factor
Naip5	Nod-like receptor apoptosis-inhibitory protein-5
S.typhi	*Salmonella enterica* serovar Typhi
S.typhimurium	*Salmonella enterica* serovar Typhimurium
E. coli	*Escherichia coli*
EPEC	Enteropathogenic *E.coli*
ETEC	Enterotoxigenic *E.coli*
EHEC	Enterohemorrhagic *E.coli*
EIEC	Enteroinvasive *E.coli*
BT	Bacterial translocation
c-di-GMP	Cyclic diguanosine monophosphate
G. xylinus	*Gluconacetobacter xylinus*
GTP	Guanosine triphosphate
DGC	Diguanylate cyclase
PDE	Phosphodiesterase
X. campestris	*Xanthomonas campestris*
csg	Curli subunit gene
IBD	Inflammatory Bowel Disease
Bcs	Bacterial cellulose synthase
C. crescentus	*Caulobacter crescentus*
P. aeruginosa	*Pseudomonas aeruginosa*
Rdar	Red dry and rough
HPLC	High-performance liquid chromatography
V. cholera	*Vibrio cholera*
IL-8	Interleukine-8
SPI	*Salmonella* pathogenicity island
TTSS	Type three III secretion system

1 INTRODUCTION

1.1 THE HOST: THE GASTROINTESTINAL TRACT

1.1.1 The gastrointestinal tract

The gastrointestinal tract (GI tract), also called the digestive tract, alimentary canal or gut, is the system within multicellular animals that takes in water and food, extracts energy and nutrients from the food, and expels the remainder as waste. Therefore, the GI tract is the major portal of entry of foreign-to-the-body compounds and organisms and is, on the other hand, connected to systemic sides in the human body. Other functions of the GI tract are the elimination of toxins, hormone metabolism and neurotransmitters production (>80%). Additionally, the GI tract is the largest reservoir of the human normal flora, which has numerous functions like competitive exclusion of pathogenic organisms, induction of immunity, breakdown of non-digestable material and production of vitamins. Over 60% of the immune system is in the GI tract, which responds to the commensal flora and intruding pathogens [1].

All the parts of the GI tract share a general structure that is referred to as mucosa. The mucosa is the innermost layer of the GI tract, surrounding the lumen, or space within the tube where digestion mainly takes place. This layer comes in direct contact with the food and is responsible for absorption and secretion. The mucosa is coated with mucus (mucus layer) that acts as a lubricant for the movement of the food through the intestinal tube. The mucosa can be divided into the epithelium, the lamina propria (connective tissue that keeps the epithelium steady) and the muscularis mucosae (thin layer of smooth muscle) [1]. Upon infection of the gut, one of the first lines of defense is the mucosal epithelium [2][3]. The mucosal cell lining of the intestine provides the largest surface area in the adult human.

The mucosal epithelium is one-cell-thick-layer mainly composed of columnar absorptive epithelial cells, but also of more specialized cells. For example, goblet cells secrete mucus; Paneth cells secrete antimicrobial molecules, e.g. antimicrobial peptides such as α-defensins; microfold (M) cells internalize microbes and deliver them to the immune cells across the epithelial barrier, and intraepithelial lymphocytes (IELs) release cytokines after exposure to pathogenic agents [2]. Epithelial cells in the small intestine are a type of brush border cell connected by tight junctions to form a polymer impermeable membrane [2] while they are are more cuboidal and compactly arranged in the large intestine.

The GI tract can be separated into upper and lower GI tract. The

lower GI tract consists of the intestine and anus. The intestine can be separated into the small and large intestine. The small intestine incorporates three features which account for its huge absorptive surface area: Mucosal folds that are circular folds, which not only increase surface area, but aid in mixing the ingesta by acting as baffles, villi that are multitudes of projections of the mucosa which protrude into the lumen and are covered with epithelial cells, and densely-packed microvilli studding the lumenal plasma membrane of absorptive epithelial cells.

The large intestine is much wider than the small and its wall is lined with simple columnar epithelium with sacculations instead of villi [1].

Closely associated with the mucosa is the immune system of the GI tract referred to as gut-associated lymphoid tissue (GALT). It includes Peyers patches (PPs), intraepithelial aggregations of lymphoid tissue, and mesenteric lymph nodes (MLN), where initial mucosal immune responses are induced [4]. In humans, Peyers patches are usually found in the most distal part of the small intestine, the ileum. Peyers patches are covered by an epithelium that contains the antigen-sampling M cells. The more diffuse effector site of GALT is the intestinal lamina propria and consists of antigen-presenting cells, including dendritic cells and subsets of T cells. In addition, at the Peyer's patches or isolated lymphoid follicles of the gut, reside B cells and plasma cells that produce intestinal IgA. This protective humoral response is the most productive immunoglobulin producing pathway in the entire body(>90%) and generates gram quantities of IgA every day [5].

1.1.2 Mucosal Immune Responses

The epithelial cell lining senses the presence of microorganisms in the lumen. When the microflora is built up after birth, intestinal homoeostasis is maintained by sensing of the commensal flora by the epithelial cell lining which generates a mild immune response preventing the overgrowth of the commensal flora. On the other hand, pathogenic bacteria are recognized and an acute immune response is triggered, which contributes to eradication of the pathogen [6]. Overgrowth of the microbial flora is prevented in various ways. A mucus layer is located on top of the epithelium, which provides a sticky mechanical barrier that protects epithelial cells. Bacteria in the mucus layer have to resist to bacteriolytic action of e.g. enzymes like lysozyme and antimicrobial peptides secreted from Paneth cells [7]. M cells sample bacteria and deliver them to the dendritic-cell-rich subepithelial area of Payers Patches for eliciting bacterial killing. Dendritic cells can also directly capture bacteria by penetrating the epithelial tight junctions and protruding their pro-

longations between the epithelial cells of the intestinal epithelium [8].

On the surface of epithelial and immune cells, the presence of the microorganisms is sensed by specific receptors, called pattern recognition receptors (PRRs), that recognize structurally conserved microbial molecules. Structurally conserved microbial structures have been termed pathogen-associated molecular patterns (PAMPs) and include lipid A part of the lipopolysaccharide (LPS) present in the outer membrane of Gram-negative bacteria, components of the bacterial cell wall such as peptidoglycan, microbial DNA and flagellin, the subunit of flagella required for bacterial motility [6][9]. Toll-like receptors (TLRs) are a group of important transmembrane PRRs. Until now, 15 TLRs have been identified, from which TLR 1-10 are found in humans [10]. TLRs recognize a broad spectrum of microbial components, e.g. TLR2 recognizes cell wall components, peptidoglycan and lipoteichoic acid [11], TLR4 the lipid A part of LPS [9][12] and TLR5 flagellin, the monomeric subunit of bacterial flagella [13]. TLRs have been found to reside on the surface or within cell compartments of, not only epithelial and innate immune cells, but also neuronal cells, endothelial cells and other cell types. After recognition of PAMPs, TLRs trigger a signaling cascade, which leads to e.g. the release of pro-inflammatory cytokines in order to promote subsequent immune responses.

Flagellin as an immunogen A PAMP that plays an important role in triggering mucosal innate immune responses, is the protein flagellin. Flagellin is the monomeric subunit that builds up the polymeric flagellar filament, which is required for swimming and swarming motility in bacteria [14][15]. Flagella are, however, also bacterial virulence factors since they are often required for bacterial colonization and tissue invasion [16][17][18][19]. The flagellar protofilament of *Escherichia coli* and *Salmonella* is almost exclusively built-up from monomeric flagellin subunits.

Flagellin carries the H-antigen specificity and is recognized as a major antigen in Crohn's disease [20]. On the surface of host cells bacterial flagellin is specifically recognized by TLR5 that leads to NF-kB activation, chemokine release, T-cell activation, and other inflammatory phenotypes. For example flagellin from *S. typhimurium* and from pathogenic and commensal *E.coli* strains, induces a proinflammatory response in gastrointestinal epithelial cell lines [21][22][23][24] and contributes to systemic inflammation in LPS-resistant mice [24]. Since epithelial cells in the gut become tolerant to LPS just after their first exposure to bacteria [25], flagellin is an important immunostimmula-

tory agent of enteric bacteria *Enterobacteriaceae* [13][26][27][28] . In the cytoplasm, interleukin-converting enzyme protease-activating factor (Ipaf) is essential for recognition of flagellin, while the Nod-like receptor apoptosis inhibitory protein-5 (Naip5) also contributes to recognition [15][29].

TLR5, a highly conserved toll-like receptor found on different cell types and in different tissues, is an important factor of flagellin-induced inflammation. TLR5 recognizes and binds only to flagellin monomers and not to polymeric flagellin, which is integral part of the flagellum [30]. Theoretically, flagellin monomers, that bind to TLR5, can have emerged from the flagellum depolymerizing at the distal end, or are secreted as monomers since they have never polymerized. Evidence so far support the latter theory, since *Salmonella* serovars Typhi and Typhimurium *de novo* synthesize and secrete monomeric flagellin after sensing of host-produced lysophospholipids during incubation with intestinal epithelial cells [31]. Synthesis and secretion of flagellin is an integral part of the flagellar filament assembly. In fact the flagellar apparatus resembles a type III secretion system. Flagellin monomers are secreted through the axial channel of the filament until its distal end, where they get polymerized in helical way [32][33][34][35]. At this point, a capping structure puts flagellin monomers into place [34][35] thus consuming the provided monomers to assemble the polymeric filament. However, there are additional ways that availability of flagellin monomers for TLR5 binding van be regulated since proteases can cleave the monomers after they are synthesized and secreted [36] while protection from this cleavage is provided by glycosylation [37]. Additionally, several bacterial pathogens use efficient mechanisms to shut-off flagellin expression within hosts [38][39][40].

The flagellin protein is a highly variable molecule. Therefore it has been used to discriminate bacteria such as *Salmonella enterica*, below the species level (H-antigen). Primary structure of flagellin can be divided to the N-terminal, the C-terminal and the central region while the tertiary structure is divided to three domains (D1-D3) [35] Fig. 1. The N-terminal and the C-terminal regions are conserved and together they form the D1 domain of the tertiary structure of flagellin [35]. According to the conservation of their N and C-terminal sequences, *E.coli* flagellins can be classified into two majors groups EC1 and EC2; the latter may be derived from the *fliC* gene of the *E. coli/Salmonella enterica* common ancestor, the former perhaps obtained by lateral transfer since species divergence [41].

Coimmunoprecipitation experiments have shown that flagellin binds

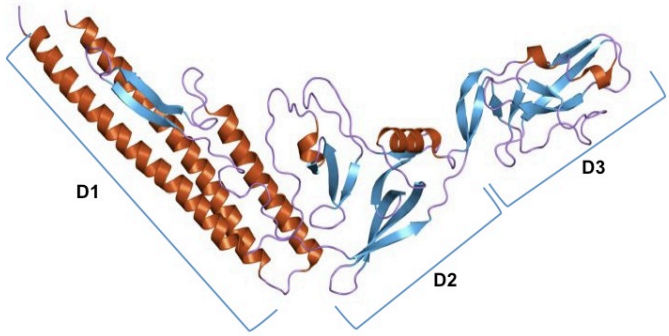

Figure 1: Flagellin monomer tertiary structure

directly to TLR5 [30]. Recognition of flagellin by TLR5 requires stretch of amino acids located in the N-and C-terminal domain of flagellin [30]. *In vivo*, TLR5 is located at the surfaces of intestinal epithelial cells towards the lumen as well as the lamina propria [42][21]. In unpolarized cell cultures TLR5 is expressed anywhere at the surface of intestinal epithelial cells, while in the case of mature polarized epithelial monolayer TLR5 resides at the basolateral side of the cells [14][43][44].The fact that mature gastrointestinal epithelial cell lines do not express TLR4 [45][46][12] renders them an ideal model to study the effect of TLR5 interactions to inflammation, where the amount of the pro-inflammatory cytokine IL-8 or transcription factor NF-kB induction, are commonly used as read-outs.

1.2 THE BACTERIA

1.2.1 Bacterial composition of the gastrointestinal tract

In contrast to the small intestine, which contains relatively few bacteria (10^5-10^7 bacteria/ml of fluid at the proximal end and 10^8 bacteria/ml at the distal end), the majority of the intestinal microbiota resides in the large intestine (10^{11}/ml feces) [47]. A huge variety of bacterial species (∼1000) inhabits the human large intestine, constituting a complex ecosystem and rendering this system a site of intense metabolic activity [48][49]. Recent metagenome sequencing [50] as well as studies with germ-free animals, [51][52][53] have given insight into the abun-

dance of bacterial species in the gut and the roles of the human gut microbial flora for human health. For example, normal flora synthesize and excrete Vitamin K and Vitamin B12 and inhibit or kill non-indigenous species through the production of nonspecific fatty acids and peroxides to highly specific bacteriocins. The normal flora also stimulates the development of certain tissues, i.e., the caecum and certain lymphatic tissues (Peyer's patches) in the GI tract, and stimulates the production of natural antibodies [54]. Generally, after birth, the first colonisers of the human gastrointestinal tract are facultative anaerobes e.g. enterococci and enterobacteria, mainly *E. coli*, [55][56] followed by obligate anaerobes [57][58]. The adult flora of the small intestine consists of bifidobacteria, enterococci, lactic acid bacteria and enterobacteria, while the flora of the colon comprises bacteroides, lactics, lactic acid bacteria, enterobacteria clostridia and methanogens [47]. The enterobacterial flora is variable and consists of transient and persistent strains; most of the strains are commensals or live in symbiotic relation with the host, but potentially pathogenic strains also colonize. Actually, *E. coli* is the predominant enterobacterial species in the gastrointestinal tracts of mammals. It accounts for 0.1% of the total bacterial biomass, which can reach up to 10^8 cells/ml [59][55][56] while the amounts of *Salmonella* in the intestine are ∼100 times less than *E.coli* [50]. Most *E. coli* strains are harmless commensals but colonization of commensal *E.coli* is found to be higher when *E.coli* pathovars are spread to susceptible sites [60][61]. It has been demonstrated that intestinal colonization of commensal *E.coli* is required for chronic intestinal inflammation [62]. Commensal *E.coli* can also cause disease through bacterial translocation in case of bacterial overgrowth due to antibiotic treatment or due to weakened immune defence of the host [63]. Bacterial translocation (BT) is the passage of viable bacteria and/or their products from the gut across the intestinal epithelium to the mesenteric lymph nodes (MLNs) and further to normally sterile organs [64]. Certain balance and composition of the commensal gut flora is important for being beneficial and health maintaining [65]. The commensal gut flora is altered under chronic inflammation conditions that characterize irritable bowel syndrome and inflammatory bowel disease (IBD) [66]. For example, the normally subordinate *E.coli*, is observed to be predominant in the case of Crohn's disease, a form of IBD [67][65]. In general, the combination of a genetic pre-disposition of the host and specific features of the bacterial flora disrupt the homeostasis between the commensal bacteria and the immune system of the host to promote chronic infection. Specifically, on the host side, epithelial barrier function, immunoregulation or bacte-

rial killing and/or processing can be disregulated [68][69]. On the other hand, bacterial virulence factors that promote adherence, invasion and persistence into epithelial cells along with bacterial metabolic products that induce epithelial injury [70] can also disrupt the homeostasis and lead to IBD pathologies.

Bacterial biofilms in the gastrointestinal tract Persistence of bacteria in the GI tract has been associated with the expression of adhesins [71][72][73]. Establishment of the bacteria can potentially have the form of a biofilm. Biofilms are matrix-enclosed bacterial populations adherent to each other and/or to surfaces or interfaces. This definition includes microbial aggregates and floccules [74]. Sessile bacteria forming biofilms in the gut are likely to play a pivotal role in gut health and disease [75][67][74][76][77][78]. In the colon, the site most heavily colonized by microorganisms, extensive biofilm formation occurs that comprises mixtures of living and dead bacteria [78]. Bacterial biofilms can provide metabolic advantages to the host, for instance, biofilm populations were found to be more efficient in digesting polysaccharides than the nonadhering bacteria, while they have distinct fermentation products [79]. Mucosal biofilms formed by commensal bacteria provide a protection barrier to the mucosal epithelium [66][80]. On the other hand, the biofilm could promote persistent colonization by protecting encased bacteria from host immune defences such as antimicrobial compounds [81][74][82][83]. Expression of adhesins and eventual biofilm formation are triggered by environmental conditions [84]. In some cases, adherence to epithelial cells is essential for bacteria in order to colonize or invade the host [72], [85]. In this context, biofilm formation can be a virulence factor like in enteroaggregative *E.coli* (EAEC) where EAEC strains adhere to the small and large bowel mucosal surface in a thick aggregating biofilm [86][87][88]. Microscopy studies have also revealed that bacteria growing on the rectal mucosa are distributed throughout the mucus layer, while most of the live cells were close to the epithelial surface [89]. This close proximity may result in localized high levels of immunogenic and toxic substances, stimulating inflammatory processes and thus resulting in disruption of the homeostasis between commensal bacteria and host's immune system leading in pathologies like IBD. Curli and other fimbriae, and the exopolysaccharide cellulose, are components of enterobacterial biofilms on epithelial cells promoting and counteracting adherence [90][91][73][92]. Additionally, the biofilm matrix component curli fimbriae mediates adherence and cytokine production and stimulate recognition of flagellin [92]. On the other hand, the switch between

a biofilm state and planktonic lifestyle is linked to virulence for some pathogenic bacteria. For example, *Vibrio cholerae* forms biofilms on zooplankton and phytoplankton in the environment, but switches to the planktonic lifestyle as soon as it enters the mammalian intestine [93][94].

Enterobacteriaceae The family Enterobacteriaceae, trivially known as enterobacteria, belongs to the phylum *Proteobacteria* and consists of rod-shaped, Gram-negative, non-spore forming, facultative anaerobes. It comprises more than 30 different genera with *Escherichia, Shigella,salmonella* and *Yersinia* as most important representatives due to their prevalence and pathogenic potential. Most of Enterobacteriaceae can be inhabitants of the intestinal tract and can also cause various diseases. Enterobacteria are responsible for foodborne disease outbreaks, which cause approximately 76 million illnesses and 5,000 deaths every year [95].

Escherichia coli *E. coli* is one of the best understood model organism. *E. coli* can be found in a variety of environments like water, fruits, manure-related soil and abiotic surfaces [96] as well as in a variety of hosts like mammals or even fish [97].Humans and animals are natural hosts of *E.coli*. Most *E. coli* are commensals, but pathogenic strains cause intra- and extraintestinal infections such as various forms of gastroenteritis, neonatal meningitis, septicemia, urinary tract infection and other severe pathologies. Distinct *E. coli* pathovars which cause intestinal infections are enteropathogenic *E. coli* (EPEC), enterotoxigenic (ETEC), enterohemorrhagic (EHEC) and enteroinvasive (EIEC). These pathovars carry distinct pathogenicity islands, which are basically accumulation of virulence factors and adhesins integrated into the chromosome, virulence plasmids and individual changes on the chromosome. Additionally, *E. coli* is also associated with inflammatory bowel disease that is a set of inflammatory conditions of the colon and small intestine. Interactions of *E. coli* with epithelial cells are studied *in vitro* with use of human cell cultures, also used for the study of *Salmonella* infection, and are described later in the *Salmonella* section (Human cell culture models)

Salmonella The genus *Salmonella* consists of 2 species, *Salmonella enterica* and *Salmonella bongori*. *Salmonella enterica* consists of 6 subspecies (group I, II, IIIa, IIIb, IV and VI) and *Salmonella bongori* is subspecies group V of *Salmonellae* [98]. According to separa-

tion of *Salmonellae* to somatic groups (O-antigens) and flagellar types (H-antigens) more than 2500 serological variants (serovars) have been described [99]. *Salmonellae* can adapt to a variety of environments and hosts but mostly live in the intestinal tracts of warm and cold-blooded animals. t is estimated that *Salmonellae* cause globally approximately 30 million human infections every year (www.who.org), resulting in 200,000 deaths [100]. The estimation results from calculation of outbreaks and unreported cases in under-developed countries, while in the USA 7000 cases were reported in 2007 [101]. Serovars able to infect mammals mainly belong to subspecies I of *Salmonella enterica*. Transmission is via a fecal-oral route, i.e., via ingestion of contaminated water or food, especially poultry and dairy products. *Salmonella* is also transmitted from person to person and secondary spread can therefore occur. Thereby, host-host restricted serovars of *S. enterica* cause systemic infections (enteric fevers) like the serovar Typhi that causes typhoid fever in humans.

Non-typhoidal *Salmonella* (NTS), among them *S. typhimurium*, are zoonotic serovars with a broad spectrum of unrelated hosts. *S. typhimurium* normally causes self-limiting gastroenteritis in immunocompetent humans, but can also cause systemic infections leading to death in immunocompromised individuals such as the elderly and pregnant women. However, *S. typhimurium* is evolving. In sub-Saharan Africa there is a dramatic increase in invasive diseases caused mainly by *S. typhimurium*. Novel variants of *S. typhimurium* arised which cause invasive disease in HIV-infected individuals [102]. In Europe, a multidrug-resistant*S. typhimurium*phage type arised which is associated with large outbreaks and increased need for hospitalization [103]. *S.typhimurium* specifically has an incubation period of 6-48h and the infectious dose is approximately 10^6 cells.

1.2.2 *Salmonella* infection

As a food-born pathogen, *S.typhimurium* must first survive passage through the acidic stomach. Then the organism adheres to the intestinal epithelium of the ileum to establish an invasive infection. Adhesion to the epithelium is multifactoral and poorly understood. Fimbrial and non-fimbrial adhesins as for instance the large, repetitive non-fimbrial adhesin SiiE, mannose-sensitive type-1 fimbriae, Lpf fimbriae and curli fimbriae have been shown to contribute to adhesion and/or disease symptoms *in vivo* or *in vitro* [104][105][106][107][91][108]. Subsequently, effector proteins of the type III secretion system-1 (TTSS-1) located on *Salmonella* pathogenicity island 1 mediate invasion of

enterocytes and M cells via an induced endocytic mechanism Fig. 2 [109][110][111][112][113][114][115][116]. *Salmonella* inside the eukaryotic cell is included within a vacuole, referred as endosome, where the bacterium multiplies. The endosome moves to the basal side of the cell, *Salmonella* are released and may be phagocytosed by macrophages. Alternativelly, crossing of the gastrointestinal epithelial wall through M cells situated in the Peyer's patches leads to penetration and distruction of the latter Fig. 2 [117]. Alternatively the bacteria are cuptured by the prolongations of dendritic cells which protrude between the epithelial cells of the intestinal epithelium Fig. 2 [8][111]. Bacteria migrate to the lamina propria of the ileocecal region where they multiply and stimulate an inflammatory response. This inflammatory response is manifested by production of pro-inflammatory cytokines, mainly IL-8 [118], which leads to recruitment of neutrophils and macrophages. Macrophages and monocytes phagocytose *S. typhimurium* and migrate to the lymphnodes [119]. There is strong influx of inflammatory cells leading to the release of prostaglandins, which activate adenylate cyclase which produces fluid secretion to the intestinal lumen thus causing diarrhea. The inflammatory response prevents the spread beyond the GI tract and eventually kills the bacteria.

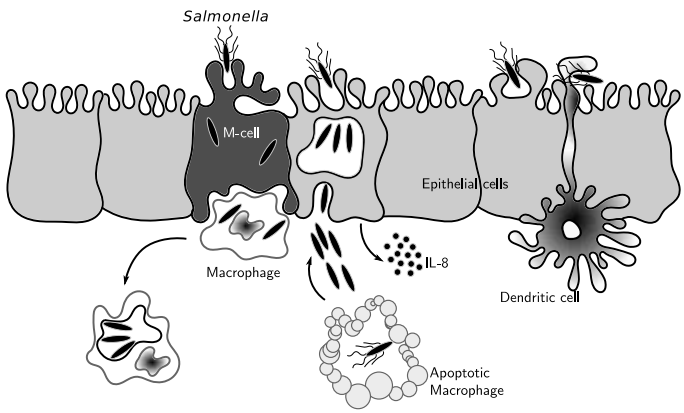

Figure 2: *Salmonella* crossing the epithelial barrier

Models for human gastroenteritis Direct information about*salmonella* infection in humans is acquired through stool samples, from intestinal biopsies or from blood analyses of patients [120][121][122]. In humans, *S. typhimurium* similarly to other nontyphoidal *Salmonella* serovars, causes a localized acute gastroenteritis, that is acute inflammation of the small intestine resulting in fever

and diarrhea with fluid and electrolyte loss, and/or lymphadenitis, that is inflammation and/or enlargement of mesenteric lymph nodes. [123][122]. *S. typhimurium* induced acute inflammation is characterized by a massive influx of neutrophils in the terminal ileum and proximal colon as revealed from patients gut biopsies [121][122], while neutrophils are also present in feces along with other fecal leucocytes, as revealed from patients stool samples [120].

Use of animal models has demonstrated that there can be significant differences between *Salmonella* pathogenesis in animals and in humans. Therefore different *in vivo* and *in vitro* models offer the study of specific pathologies under specific conditions, adding to different aspects of*Salmonella* infection.

Domestic food-producing animals like calves [124], sheeps [125], pigs [126] and poultry [127] are natural hosts of *S. typhimurium* causing enterocolitis with similar pathologies to humans. Bovine colitis is consequently a good model to reflect human enterocolitis [124][128]. However, cattle are usually outbred and their size and cost restrict their use. Rabbits, on the other hand, are well established inbred animals where oral infection with *S. typhimurium* results in systemic infection [129].

Injection of*S. typhimurium*into ligated ileal loops of animals is a model that is used to study the early events of infection up to six hours. *S. typhimurium*injection into ligated ileal loops of calves or rabbits results in intestinal inflammation and fluid accumulation, pathologies that mimic infection via the natural oral route. The corresponding murine ligated loop *S. typhimurium* infection model demonstrates milder inflammation [130], but still is a good model to study early interactions of*S. typhimurium*with intestinal epithelial cells *in vivo* and to confirm observations from tissue culture experiments [131][128].

Inbred strains of mice vary in their sensitivity to serovar Typhimurium infection, from being relatively resistant (oral $LD_{50} \geq 10^8$ bacteria) to highly sensitive (oral $LD_{50} \geq 10^4$ bacteria). The natural resistance is mediated by a single locus on chromosome 1 called Nramp1 [132] that is almost exclusively expressed by macrophages. Oral infection of *Salmonella*-susceptible mice with serovar Typhimurium results in a systemic disease with bacteremia and lesions in systemic organs, mouse typhoid fever, that resembles the infection of *S. typhi* in humans. Consequently, this model is frequently used as an experimental animal model to study typhoid fever [131].On the other hand, in genetically resistant inbred mouse strains (e.g. 129SvEv; Nramp1+/+)*S. typhimurium*causes chronic infection of systemic organs. *Salmonella* can only poorly colonize the intestine, a fact that is referred to as "col-

onization resistance" of the murine intestine. In germ-free or antibiotic treated mice, colonisation resistance is abolished. Streptomycin treatment prior to *S. typhimurium*infection disrupts the colonization resistance and results in acute inflammation in the intestine with neutrophils influx and epithelial erosions [133][134] mirroring human *S. typhimurium* infections. However, *Salmonella* infection does not result in diarrhea and longterm infection is accompanied by systemic spread of bacteria; properties that are not characteristic of human *S. typhimurium* infections. A functional gut flora is required for colonization resistance, as re-association of germ-free mice with commensal bacteria restores colonization resistance [54].

Human cell culture models are used to investigate molecular mechanisms leading to bacterial virulence phenotypes and changes in host cell gene expression. Human colon carcinoma cell lines such as T84, CaCo-2 and HT29 are commonly used to study early interactions of *Salmonella* and *E.coli*, with the intestinal epithelial lining such as adherence, invasion, replication in epithelial cells, induction of proinflammatory immune response and bacterial translocation by using the transwell system. Transwell culture allow polarized growth of the cells providing, for example, an intact apical brush border or co-culturing with immune cells, e.g macrophages or dendritic cells [135][8]. One limitation, however, of these cell lines is their cancerous nature. Normal small intestinal cell lines are also used, such as the HIECs [136], a series of human intestinal cell lines with typical crypt cell proliferative characteristics, the tsFHIs [137], a set of conditionally immortalized fetal human intestinal cells and the PCDEs [138], which are fully differentiated enterocytes that can be maintained in primary culture for about 10–12 days.

Other non-intestinal immortal epithelial cell lines like HeLa or Hep-2 cell lines have also been used to study interactions of *S.typhimurium* with epithelial cells. Additionally, tissue explants are used to study the *S. typhimurium* -intestine interactions [139][140].

***Salmonella* Pathogenicity island 1 (SPI-1)** *Salmonella* Pathogenicity island-1 (SPI-1) is a ∼40kb region of the *Salmonella* genome that encodes the 39 proteins of a prokaryotic type three secretion system (TTSS-1).Two operons *srg/org* and *inv/spa* are required to build up a syringe-like apparatus that streches from the inner membrane over the outer membrane into the extracellular space [141][142][143]. In addition, SPI-1 also codes for most of the effector proteins translocated into the eukaryotic target cell by TTSS-1 and SPI-1 regulatory

proteins. SPI-1 was acquired after *Salmonella* separated from *E. coli*, as the island is inserted between two adjacent genes from *E.coli* K-12 [145]. The island seems to be an ancient acquisition since is present in all *Salmonella* subspecies [144]. TTSS-1 is required for the initial steps of *Salmonella* pathogenesis [148][149][113]. TTSS-1 is necessary for invasion of *Salmonella* in non-phagocytic epithelial cells [146][147]. Thereby, TTSS-1 is a syringe-like apparatus translocates effector proteins to the host epithelial cell in order to induce host-cell membrane ruffling and consequent internalization [150][151][152][141]. The concerted action of effector proteins encoded by SPI-1 such as AvrA, SipA, SipB, SipC, SipD, SlrP, SptP and SspH1 or outside the SPI-1 locus (SopA, SopB, SopD, SopE and SopE2) [112][143][109][153], results in invasion of *Salmonella* into non-phagocytic epithelial cells [109]. Effector proteins are translocated in a time-depenent way. The sipABCD contributes to invasion since SipB/SipC/SipD translocates effector proteins to the host cell, while SipA and SipC induce host-cell actin re-arrangement by nucleating and bundling F-actin filaments [113][110][154][115][143]. On the other hand, SopE and SopE2 activate the Rho family GTPases Cdc42 and Rac1 to induce actin re-arrangement and *Salmonella* uptake in the epithelial cell [155][146]. The inositol phosphate phosphatase SopB [156], works together with SopE and SopE2 to contribute to membrane ruffling and bacterial uptake [110][112]. SptP, on the other hand, inactivates Cdc42 and Rac1 thus terminating epithelial uptake and reestablishing an intact host cell [157]. Additionally to their requirement for the invasion process, SPI-1 TTSS secreted effector proteins induce fluid accumulation, polymorphonuclear cell infiltration, and expression of pro-inflammatory chemokines [158][159].

TTSS-1 regulation Virulence regulation is spatially and temporally highly coordinated. Optimal SPI-1 TTSS expression requires high osmolarity, low oxygen and slightly basic pH; conditions as present in the small intestine [160][161][149][143]. Transcriptional regulation of SPI-1 TTSS genes involves a number of regulators encoded within the SPI-1 or outside. *hilA* encoded within SPI-1, is the central transcriptional activator required for the expression of SPI-1 TTSS [162][163]. *hilA* contains a DNA binding motif of the OmpR/ToxR family and binds directly to promoters to activate expression of the *prg/org*, and *inv/spa* operons. Subsequently, *hilA* activates the positive transcriptional activator, InvF, encoded by the first gene in the *inv/spa* operon, which in turn activates, together with the chaperon SicA the SPI1 TTSS secreted effector proteins.

HilA expression is coordinatively regulated by the AraC-like transcriptional activators HilC, HilD and RtsA, encoded outside of SPI-1 [149][164]. These regulators individually bind to the *hilA* promoter and act in complex feed-forward loop with transcription of *hilA* as the output [160][161]. They can also act independently of *hilA*, for example by inducing expression of effector protein SlrP and DsbA, a protein needed for TTSS functionality. Additionally, TTSS-1 effector genes that are not on SPI1, SopB and SopE, are expressed from InvF-dependent promoters [148].

Several global regulatory systems, widely distributed among pathogenic bacteria and encoded outside SPI-1, regulate the expression of SPI-1 TTSS mainly through HilD. Such global regulatory systems include the BarA/SirA and OmpR/EnvZ two-component systems [143][137][165][164]. The ferric uptake regulator (Fur) regulates *hilA* transcription by controlling translation of the positive regulator HilD. Furthermore, FimZ regulators of type 1 fimbriae genes and the two-component system PhoP/PhoQ, also modulate SPI-1 expression by modulating *hilE* expression which is a negative regulator of HilD.

1.3 C-DI-GMP SIGNALING

1.3.1 The second messenger c-di-GMP

A biological response to an intra- or extra-cellular signal, the first messenger, is rapidly locally or generally amplified through e.g. enzymatic metabolic changes in the concentration of the second messenger. As a consequence, binding of the second messenger to a receptor (effector) is altered which leads to an alteration of the target.

Cyclic-3'5'-diguanylic acid (c-di-GMP) Fig. 3 is a cyclic dinucleotide first identified more than twenty years ago as the allosteric activator of membrane-bound cellulose synthase in the fruit-degrading bacterium *Gluconacetobacter xylinus* (previously called *Acetobacter xylinum*) [166]. Only recently, it has been recognised as a bacterial secondary messenger [167][168] since it has shown to have a more global role as a signaling molecule in bacteria. C-di-GMP is almost ubiquitous among bacterial species, but is exclusively found in bacteria, not in archeae and eukaryotes [169][170][171][172]. The change of concentration of the second messenger c-di-GMP takes place through the action of diguanylate cyclases (DGC) and phosphodiesterases (PDE) that are responsible for the biosynthesis and hydrolysis of c-di-GMP. C-di-GMP has been shown to bind to a variety of receptors (proteins and riboswitches) with the consequence of physiological changes. C-di-GMP signaling contributes

to the regulation of a wide spectrum of phenotypes. The most investigated phenotypes are motility, sessility and virulence. These bacterial phenotypes are often interconnected as they promote or inhibit each other.

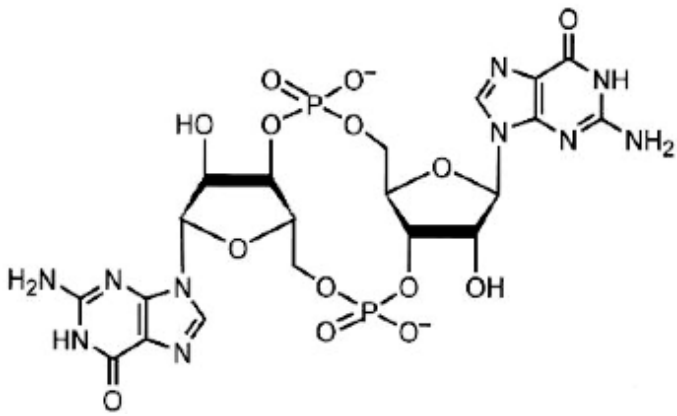

Figure 3: Cyclic-3'5'-diguanylic acid (c-di-GMP)

1.3.2 C-di-GMP metabolism - GGDEF and EAL domain proteins

Synthesis of c-di-GMP is catalyzed by GGDEF domain proteins acting as di-guanylate cyclases. On the other hand, EAL or HD-GYP domain proteins [173] act as c-di-GMP specific phosphodiesterases Fig 4 [169]. C-di-GMP is synthesized from two molecules of GTP, via the intermediate substrate linear diguanylate triphosphate pppGpG, with the concurrent release of two phosphates (PPi). Consequently two more phosphates are released from linear pppGpG, to form c-di-GMP [174]. Degradation of c-di-GMP occurs by hydrolysis resulting in the linear di-nucleotide pGpG in the case of hydrolysis by EAL-domain PDEs. Hydrolysis by HD-GYP-domain PDEs results in two pGs [175].

GGDEF and EAL domain proteins are widespread in bacterial genomes [169][170][171][172]. Very often one bacterial genome contains more than one GGDEF and EAL domain protein raising the question of specificity of the c-diGMP signaling pathway(s). The sequenced genome of*S. typhimurium*codes for 20 GGDEF/EAL domain proteins; 5 contain a GGDEF, 8 an EAL domain and 7 contain both. On the other hand, *E. coli* K-12 has 12 GGDEF, 12 EAL and 7 GGDEF-EAL domain proteins. The first characterization of DGCs and PDEs in *G.xylinus* revealed two conserved domains, GGDEF and EAL named after characteristic highly conserved amino acid residues [169].

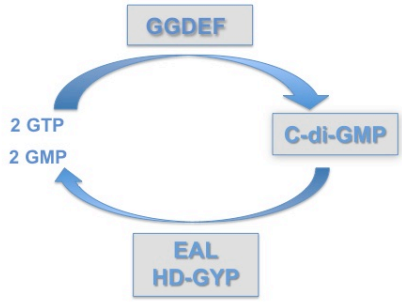

Figure 4: C-di-GMP metabolism by GGDEF/EAL domain proteins.

Genetic and biochemical analysis of GGDEF domain proteins revealed that the GGDEF domain is responsible for the diguanylate cyclase activity [176][174][177][178][179][180]. In general, GGDEF domains are approximately 170 amino acids long [170]. The GG(D/E)EF motif is an integral part of the active site and mutation of any residue of the motif abolishes enzymatic activity [174][180]. Stand-alone GGDEF domains are usually not enzymatically active, but require activation through an N-terminal signaling domain for activation. Structural analysis of the GGDEF-domain protein PleD in *Caulobacter crescentus*, showed that PleD dimerizes to catalyze c-di-GMP synthesis [174].

Most GGDEF domains contain an RxxD motif, named the inhibitory I-site, N-terminal of the active site binding dimeric c-di-GMP allosterically inhibits DGC activity. This noncompetitive product inhibition limits the concentration of c-di-GMP produced by the respective DGC. As a physiological consequence, c-di-GMP binding to the I-site is suggested to prevent the depletion of the GTP pool [181].

The EAL domains are approximately 250 amino acids long. The EAL domain requires $Mg2^{+}$ or $Mn2^{+}$ for activity, but it is strongly inhibited by $Ca2^{+}$ or $Zn2^{+}$. The glutamic acid of the EAL motif participates in $Mg2^{+}$ coordination [170]. Besides the EAL motif, there are several other highly conserved motifs involved in catalysis, substrate binding and di-valent ion coordination. Usually, EAL domains show significant enzymatic activity without N-terminal allosteric activation [183].

When proteins contain both, a GGDEF and EAL domain, both domains can be enzymatically active [171]. Alternatively, only one domain possesses enzymatic activity, while the enzymatically inactive domain can serve a regulatory function [182][183]. As a third alternative, there is the possibility that none of the domains has enzymatic activity or proteins with only one of the domains don't work neither as cyclases or phosphodiesterases [184].

GGDEF or EAL domain proteins often contain additional sensory and signal transduction domains such as PAS, GAF, HAMP REC, and HTH domains [170]. It has been shown that oxygen, amino acids, electrons, and photons can modify the activity of DGC or PDE proteins [168][167]. For example, PAS is a conserved protein domain involved in sensing oxygen, redox or light and REC (or CheY) is a chemotaxis response regulator domain that participates in signaling phosphorelays. PleD, for example, is a GGDEF domain protein that carries also the REC domain that gets phosphorylated of it's conserved aspartate by cognate histidine kinases while GGDEF is the output (effector) domain that catalyzes c-di-GMP synthesis [170][180]

C-di-GMP metabolizing proteins have been shown to be localized supporting the general concept of localized amplification of the c-diGMP signal by individual GGDEF/EAL domain proteins responsible for subsequent physiological changes. In *G.xylinus* the DGCs and PDEs affecting cellulose biosynthesis, the c-di-GMP target cellulose synthase and most of the intracellular c-di-GMP are located in the membrane fraction [169][185] implying that c-di-GMP is concentrated in distinct membrane signal-receiving niches. In *C. crescentus*, the di-guanylate cyclase PleD, becomes localized to one cell pole after phosphorylation and activation [180]. FimX, a protein from the nosocomial pathogen *Pseudomonas aeruginosa*, which contains both GGDEF and EAL domains in addition to CheY and PAS signaling domains, is found localized at the cell pole [196].

1.3.3 C-di-GMP receptors

Several receptors for c-di-GMP are subsequently detected [186][187][185]. Activation of cellulose biosynthesis was the first phenotype found to be activated by c-di-GMP [166] whereby c-di-GMP binds to the PilZ domain of the cellulose synthase BcsA in *G. xylinus*, *S. typhimurium* and *E. coli* [180][181][186][187]. The PilZ domain is a c-di-GMP binding domain, widespread in bacteria. The PilZ domain is not only present in bacterial cellulose synthases, but in a wide variety of proteins that regulate different phenotypes such as alginate

production, virulence and motility.

1.3.4 C-di-GMP regulatory targets-Implication of c-di-GMP in various phenotypes

Regulation of sessility by c-di-GMP is complex and occurs at various levels. In general, positive regulation of exopolysaccharide production, biosynthesis of adhesive fimbriae and biofilm formation by c-di-GMP is a common feature of bacterial species with community behaviors and adhering properties [188][189][190]. In *S. typhimurium*, for example, c-di-GMP synthesized from di-guanylate cylcase AdrA [191] is thought to bind to the PilZ domain of the cellulose synthase BcsA and thereby allosterically control cellulose production and the associated pdar (pink, dry and rough) morphotype present when agar-grown bacteria express cellulose. Overexpression of AdrA resulted in increased c-di-GMP levels and increased pdar morphotype. On the other hand, overexpression of the c-di-GMP dependent phosphodiesterase YhjH, a stand-alone EAL domain protein, resulted in decreased c-di-GMP levels and decreased pdar phenotype. In addition to cellulose biosynthesis, c-di-GMP regulates the expression of the biofilm transcriptional activator CsgD and subsequently CsgD-controlled genes encoding biofilm matrix components such as the *csgBA* gene encoding curli fimbriae [192], *bapA* coding for the large surprotein BapA [193] and *yih* for O-antigen capsule [194].

Motility is commonly negatively regulated by c-di-GMP in various bacteria [177][195][196][187][190]. Thereby, various types of motility, including flagella-mediated swimming and swarming, but also type IV pili mediated twitching motility are affected by c-di-GMP. First experiments, in *S.typhimurium*, have shown that high levels of c-di-GMP generated by overexpression of the DGC AdrA inhibited swarming and swimming motility while reduction of c-di-GMP levels by overexpression of the PDE YhjH stimulated motility [177]. Recently, the molecular basis of c-di-GMP mediated inhibition of swimming motility starts to become resolved. C-di-GMP binds to the PilZ domain protein YcgR leading to a conformational change in the protein [186][187]. Consequently c-di-GMP loaded YcgR can for a complex with FliG and FliM that are part of the flagella rotor. This interaction causes a back-break and the bacterium to slow-down [**?**]. C-di-GMP is affecting motility negatively also in the gastrointestinal pathogen *Vibrio cholerae*. In this bacterium, overexpression of the DGC VCA0956 or mutation of the PDE VieA, increased concentration of c-diGMP that directly binds to CsgD-like transcriptional activator VpsT [190] and abolished swimming motility by repression of genes involved in flagellum biosynthesis, motil-

ity, and chemotaxis [197].

In *C. crescentus* cyclic di-GMP signaling is implicated in developmental transitions, since DGC activity of PleD is needed for ejection of the flagellum, stalk formation, and synthesis of the holdfast [198]. Besides sessility and motility, c-di-GMP signaling affects other pathways. It is also involved in regulating e.g. the resistance to phage infection and heavy metal ions in *E. coli* and in photosynthesis in *Synechococcus elongatus* [199][200][188].

1.3.5 C-di-GMP in virulence

C-di-GMP signaling is involved in virulence of human, animal and plant pathogens like *S.Typhimurium*, *Vibrio cholerae*, *Pseudomonas aeruginosa*, *Bordetella pertussis*, *Xanthomonas campestris*, *E.coli*, *Legionella pneumophila*, *Brucella melitensis* and *Anaplasma phagocytophilum* [201][202][197][198][199][200][174][203][204][205][206][207][208]. In the gastrointestinal pathogen *V. cholerae* downregulation of c-di-GMP levels leads to activation of cholera toxin [202][209][210] More specifically the phophodiesterase VieA reduces c-di-GMP concentration inducing maximal expression of the cholera toxins through their transcriptional activation by ToxT, a direct activator of ctxAB encoding cholera toxin. VieA is part of the three component system VieSAB suggested being activated upon entry to the host [211][94]. CdpA GGDEF-EAL domain protein acting as a phosphodieterase under the control of its degenerate GGDEF domain also support the general scheme that c-di-GMP must be down-regulated after entering the small intestine [212]. Additionally, mutation of CdgC resulted in increase of transcription of *tcpA* which consequently regulates ToxT [213]. In the nosocomial pathogen *P. aeruginosa* c-di-GMP signaling is required for biofilm formation [189], a virulence phenotype of this bacterium in chronic infection. Acute virulence phenotypes are also affected by c-di-GMP signaling in *P. aeruginosa*. However, the subset of GGDEF/EAL mutants demonstrating an alteration of the cytotoxic phenotype in CHO cell line was only partially overlapping with the subset contributing to virulence in a burn wound mouse model. In general, the common view arised that c-di-GMP is promoting chronic infections, while inhibiting acute infections [214]. The plant pathogen *Xanthomonas campestris* pathovar campestris (Xcc) can cause disease through expression of RpfG, a HD-GYP domain protein that responds to diffusible signaling factor (DSF) to control *Xcc* virulence traits like production of extracellular enzymes and extracellular polysaccharide and motility. DSF signalling by interaction of RpfG with two GGDEF-domain proteins, control motility

[204][215]. In *Bordetella pertussis* a genetic screen had indicated the contribution of c-di-GMP signaling proteins to virulence [216] whereas the EAL domain protein is BvgR encoded by the gene *bvgR* that is part of the *bvgASR* locus that controls expression of *B.pertussis* virulence factors, found to be a repressor of gene expression. Mutation of *bvgR* resulted to high expression of the *bvg*-repressed genes that subsequently resulted to attenuation of disease in the mouse aerosol model [203].

In *S. typhimurium*, the EAL-domain like protein STM1344 causes resistance to *Salmonella* induced oxidative stress, inhibits rapid macrophage killing and is required for virulence in the typhoid fever mouse model [201]. STM1344, however, does not possess phosphodiesterase activity and also does not bind c-di-GMP [217], therefore the involvement of c-di-GMP signalling in these phenotypes remains elusive. Another study did not find a role for c-di-GMP in typhoid fever virulence, as the loss of virulence in a*S. typhimurium*strain, with deletion of all the GGDEF domain proteins was recovered by a single GGDEF domain protein independently of c-di-GMP synthesizing activity [218]. However, there are indications from other studies of a role of c-di-GMP in *S. typhimurium* virulence. Survival of *Salmonella* in pigs required the GGDEF-EAL domain protein STM1703 [219]. Biochemical analysis of the STM1703 homologue of *E. coli* has been shown to display c-di-GMP specific PDE activity. Additionally, flooding of the cell with c-di-GMP, resulting from overexpression of GGDEF-domain protein AdrA, led to loss of invasiveness and pro-inflammatory immunogenicity [220].

2 METHODS

The general principles and experimental set-ups used in the papers and manuscripts of this thesis are presented in this section. Detailed description of the protocols is provided in the Materials & Methods parts of the respective papers and will not be repeated here.

2.1 MOLECULAR BIOLOGY METHODS

2.1.1 Construction of mutants

Mutations in the chromosome of *S. typhimurium* and E. coli were generated in order to investigate the contribution of a gene and the corresponding encoded protein to a certain phenotype. For deletions of genes the Datsenko and Wanner method was used [221]. This fast and efficient method directly replaces the bacterial gene with an antibiotic resistance gene by means of homologous recombination using a linear DNA fragment which carries a selective marker flanked by sequences homologous to the gene of interest. In total three proteins of the lambda red recombinase complex allow not only the transformation of bacteria with linear DNA, but also recombination of two DNA sequences with sequence homology of as little as 36-50 nucleotides. The antibiotic resistance cassette is flanked by flippase (Flp) recognition target (FRT) sites allowing removal of the resistance cassette by Flp recombinase, if required. Transfer of a mutant allele with a selectable marker into a novel strain background was carried out using phage transduction with bacteriophage P22 HT105/1 *int-201* as the transducing agent.

2.1.2 Reporter Fusion protein

Fusion proteins are proteins created through the joining of two or more genes which originally code for two proteins, the protein to be monitored and the reporter protein. Translation of the gene fusion ideally results in a single polypeptide with functional properties identical to the individual proteins. Certain proteins are chosen as reporters because the characteristics they confer on organisms expressing them are conveniently identified and measured and interference with other proteins or pathways is low. Proteins usually used as reporters are the β-galactosidase, the green fluorescent protein and β-lactamase. In Paper I, the reporter fusion protein SopE2- β-lactamase (SopE2-TEM-1) was created to measure the secretion of the effector protein SopE2. β-lactamase was chosen as a reporter, as it has been demonstrated before that GFP fusion proteins are not transported by the TTSS. In its basic

function, the β-lactamase catalyses the hydrolysis of β-lactams, a class of antibiotics that target cell-wall biosynthetic enzymes located in the periplasm. Therefore, β-lactamase must be exported beyond the cytoplasm to be active. For this reason, β-lactamase is an export reporter with enzymatic cleavage of β-lactam as a powerful indicator of export. In our case, the substrate of the β-lactamase was the chromogenic β-lactam nitrocefin. Hydrolysis of nitrocefin by the β-lactamase results in a color change from yellow to red which is used to monitor the level of SopE2 secretion. The blaM gene encoding the TEM-1 b-lactamase is expressed in pCX340 plasmid under the control of the IPTG-inducible Ptrc promoter [222]. The gene encoding the TTSS-1 effector protein SopE2 amplified from *S. typhimurium* UMR1 genomic DNA was cloned upstream of *blaM* to generate a SopE2-TEM-1 fusion protein (plasmid pAPL1).

2.1.3 Sequence analysis

To investigate the molecular basis of the properties of a protein, the corresponding gene encoding the protein to be investigated can be sequenced. Translation of the gene sequence provide the protein sequence. In Paper III the molecular basis of the immunogenicity of flagellin proteins was investigated by sequencing *fliC*, the gene encoding flagellin, from the strains investigated. Sequencing was performed with primers up- and down-stream of *fliC* as well as primers inside the gene to create a set of overlapping DNA segments resulting to a *fliC* contig for each strain investigated.

2.2 INFECTION BIOLOGY METHODS

2.2.1 Cell culture model of infection

Invasion assay The human adenocarcinoma epithelial cell line HT-29 was used for the *in vitro* studies. In order to monitor the first line of events during infection with *S. typhimurium* bacteria were co-incubated with HT-29 epithelial cells for only one hour. Before co-incubation bacteria had been grown under invasion inducing conditions (described in Paper I and II). One hour post infection, supernatant was removed, cells were gently washed and cell culture medium containing gentamicin was applied to the cells for 1 h to kill extracellular bacteria. Cells were afterwards gently washed and then disrupted to release intracellular contents. The number of intracellular bacteria was determined by cfu counts of viable bacteria.

Stimulation of human epithelial cells In order to investigate the immunostimulatory capability of different bacterial strains or purified flagellin, co-incubation with HT-29 cells was performed. Undifferentiated HT-29 cells do not differentiate in apical and basolateral side and express TLR-5 receptor homogenously at their cell surface being a convenient tool to study induction of pro-inflammatory response due to TLR-5. Bacteria or purified flagellin were applied to the cells and supernatants were collected. Supernatants were centrifuged to avoid interfering of cells with the measurement of interleukine, and then analyzed for production of pro-inflammatory IL-8.

2.2.2 Animal models of infection

Prior to infection, bacteria were grown under invasion inducing conditions.

Ileal loop infection model Invasion *in vivo* was examined by using the ligated ileal loop infection model [223] that is is used to study the first events during bacterial infection of intestinal epithelial cells. *S. typhimurium* inoculum is injected directly into ligated ileal loops of anaesthetized mice. After incubation of the loop for 90 min in mice, extracellular bacteria is removed by washes with (phospahte buffer saline)PBS and adhering bacteria are killed by incubation in gentamicin solution for 90 min. Following, the whole tissue is mechanically disrupted and appropriate dilutions of the homogenized samples are spread on LB plates with appropriate antibiotics to determine the number of intracellular bacteria. This models mimics the pathology seen in the small intestine following infection via the natural oral route. In contrast, oral administration of *S. typhimurium* in mice results in typhoid fever (typhoid fever model). So that model strictly monitors the interactions of bacteria with intestinal epithelial cells and not their access to them, neither their fate during the next steps of infection.

Streptomycin-pretreated mouse model Streptomycin-pretreated mouse model is used in Paper II to study the ability of *S. typhimurium* strains to colonize and persist in the intestinal tract. This animal model is an established model for Salmonella-induced colitis [133]. 129Sv/Ev mice were pre-treated by gavage with streptomycin. 129Sv/Ev are naturally resistant to *S.typhimurium* infection (Nramp1+/+) and they are used to be able to withstand longterm infection. Streptomycin pretreatment clears the normal flora of the

mice and disrupts colonization resistance resulting in acute in acute inflammation in the intestine (neutrophils influx, epithelials erosion) after infection with *S. typhimurium*. 24 h after streptomycin pretreatment, the mice were intragastrically inoculated with bacteria. Fresh fecal pellets were collected from individual mice aseptically every second day, starting on the first day after infection and for a period of 28 days. Fecal weight was determined and feces were suspended in PBS. Serial dilutions for plating were made in PBS and plated on *Salmonella* selective medium (MacConkey) agar plates as described [133] for bacterial enumeration.Tissue samples from the mesenteric lymph node, spleen and liver were removed aseptically and homogenized bacterial loads were determined by plating the homogenised tissue samples on MacConkey agar plates.

2.3 PROTEIN METHODS

2.3.1 Detection of secreted proteins

In order to investigate secreted effector proteins and secreted flagellin proteins were precipitated from culture supernatants. Strains were grown under invasion-inducing conditions to mimic conditions of infection. Bacteria were removed by repeated centrifugation and the proteins were precipitated from the supernatant with trichlooacetic acid (TCA). Cell-associated proteins were recovered from bacterial pellets. and analyzed along with precipitated secreted proteins by SDS-PAGE and Western blot.

2.4 ANALYTICAL METHODS

2.4.1 HPLC

High-performance liquid chromatography (HPLC) is used for seperation of the components of a liquid solution, passing it through a chromatographic, with the assistance of high pressure pumps. In HPLC there are two phases: a) The stationary phase that is composed (packing material) from solid porous material , or liquid mounted on solid substrate of very small diameter, that is in the column. b)The mobile phase that is a solvent or mix of solvents. The transfer of the liquid mobile phase through the stationary,is performed using high pressure pumps and thus achieve difficult separations in minutes. It is especially useful for separation and analysis of mixtures of molecular, or ionic compounds with low vapor pressures and thermally unstable compounds, which can not be purged without break and also, in contrast with gas chromatography

(GC), is used to separate mixtures of substances with high molecular weight and polarity. For the determination of c-di-GMP nucleotides extracts [224] were subjected to ion pair chromatography using a Hypersil ODS C18 column as previously described [224]. C-di-GMP eluted at 21 min as determined by a spiked sample. The concentration of c-di-GMP in the samples was estimated from the peak area using an extinction coefficient of e = 11 800 at 254 nm.

3 RESULTS AND DISCUSSION

3.1 PAPER I

Cyclic di-GMP signalling controls virulence properties of *Salmonella enterica* serovar Typhimurium at the mucosal lining.

C-di-GMP is a global secondary messenger in bacteria that promotes physiological changes as response to environmental cues which change the intracellular levels of c-di-GMP. In *S. typhimurium*, c-di-GMP signaling has been shown to regulate bacterial behavior such as motility and sessility [171][177][168]. In pathogens, motility and sessility behaviour contribute to certain virulence phenotypes [214][225][226]. In addition, c-di-GMP signaling has been demonstrated to play a role in the regulation of virulence in various pathogens [201][202][197]. In *S. typhimurium*, the EAL-like protein STM1344 has been demonstrated to mediate resistance to hydrogen peroxide and to delay macrophage killing [201]. However, STM1344 does not metabolize or bind c-di-GMP [217], thus the contribution of c-di-GMP to virulence phenotypes of *S. typhimurium* was not clear.

Therefore the aim of Paper I and II was to study the potential role of c-di-GMP in bacterial-host interactions with the enteric pathogen *S. typhimurium* as a model organism. To this end, we wanted to detect the effect of c-di-GMP signalling on virulence phenotypes *in vitro* and*in vivo* and identify which components c-di-GMP signaling affects to control virulence.

The facultative intracellular pathogen *S. typhimurium* must adapt from an extra-host life style to growth conditions in the host [227]. The sequence of events during the infection process of *S. typhimurium* is very well characterized [141][228][114][110][143], One of the first line of defence is the intestinal epithelium, where *S. typhimurium* invades the epithelial cells and causes a pro-inflammatory cytokine response [116][228][118]. Both events contribute to acute inflammation, which is the manifestation of *S. typhimurium* enterocolitis in humans. For the *in vitro* studies, consequently, invasion of the gastrointestinal epithelial cell line HT-29 and induction of a pro-inflammatory response in the gastrointestinal epithelial cell line HT-29 were chosen as representative phenotypes to monitor virulence of *S. typhimurium*. These two phenotypes are very well characterized on the molecular level [14][43][44][112][153][229][148].

In order to demonstrate an effect of c-di-GMP signaling to virulence phenotypes, we saturated the cell with c-di-GMP through overexpres-

sion of the GGDEF domain protein AdrA, an efficient di-guanylate cyclase. In previous studies of the Römling group, overexpression of AdrA significantly increased the c-di-GMP levels in *S. typhimurium* under all conditions studied [177][230](unpublished results). Similarily, we show in paper I that AdrA expressed under invasion conditions produced significant amounts of c-di-GMP. Therefore, it was possible for us to study the change in physiology and behavior of the bacterium in the high c-di-GMP situation under the virulence challenge.

In Paper I we demonstrated that high amounts of c-di GMP in *S. typhimurium* lead to a pronounced inhibition of invasion of the bacteria into epithelial cells and to inhibition of IL-8 production from epithelial cells after infection. In fact, both virulence phenotypes of *S. typhimurium* were set back to the level of the negative control. The effect of c-di-GMP in *S. typhimurium* is consistent with findings in the gastrointestinal pathogen *V. cholerae* where high c-di-GMP levels created by mutating the phophodiesterase VieA, inhibited transcription of the transcriptional regulator ToxT and consequent production of cholera toxin, thus aborting virulence of *V. cholerae* [202][209][210]. Subsequently, we analysed through which components c-di-GMP acts to cause inhibition of invasion. Proteinaceous curli fimbriae and the exopolysaccharide cellulose are major extracellular matrix components of *S. typhimurium* plate-grown biofilms. The production of these extracellular matrix components is stimulated by c-di-GMP [231][177][187][168][232]. Cellulose and curli fimbriae and other extracellular matrix components were already shown to play a role in pathogen-host interaction in a variety of bacteria [90][91][73][92]. In addition, previous studies had shown that *S. typhimurium* has the ability to form biofilms on intestinal epithelial cells, whereby, among other components, the biofilm matrix components cellulose and curli fimbriae are required for biofilm formation [90][73]. Curli fimbriae and cellulose production requires the transcriptional activator CsgD [233][192][191]. Other extracellular matrix components regulated by CsgD, which is itself activated by c-di-GMP signaling [192],are the large surface protein BapA [193] and the O-antigen capsule [194]. Indeed in Paper I we showed that c-di-GMP inhibits invasion through genes required for the production of extracellular matrix components cellulose (through *bcsA*, the cellulose synthase) and capsule (through *yihQ*). At the same time, however, the proteinaceous curli fimbriae enhanced invasion suggesting that not all biofilm matrix components are functionally similar, but the effect on invasion is dependent on the nature of the component. Generally, exopolysaccharides interfere with invasion and type III secretion system

functionality as shown for the LPS O-antigen and for the Vi-capsule in *S. typhi* [234][235][236][152][237]. Probably, expopolysaccharides promote inter-bacterial adherence and counteract invasion that requires attachment via proteinaceous matrix, and TTSS injection of effectors. On the other hand, proteinaceous curli fimbriae mediate invasion of epithelial cells [92][85].most likely through attachment, since attachment of the bacteria to epithelial cells mediates docking of the TTSS to the epithelial cell and subsequent secretion of effector proteins and invasion [238].

In Paper I, we additionally show that c-di-GMP mediates inhibition of invasion of epithelial cells through the biofilm regulator CsgD. This effect can be attributed to the positive effect of CsgD on cellulose and capsule production [239][194]. However, cellulose production can occur independently of CsgD [233][240], since alternative di-guanylate cyclases can produce the c-di-GMP that binds to the PilZ domain of the cellulose synthase BcsA. Indeed, deletion of the cellulose synthase had an additional effect on restauration of invasion in the *csgD* mutant. Whether CsgD activated cellulose biosynthesis is involved in inhibition of invasion could not be resolved in the experimental setting used. However, CsgD contributed to reduced secretion of the SPI-1 TTSS effector protein SopE2 independently of production of CsgD-regulated extracellular matrix components, implying that an unknown component regulated by CsgD interferes with TTSS secretion of effector proteins. As SopE2 secretion was monitored using a plasmid with an inducible promoter, the effect of c-di-GMP and CsgD is most likely beyond SpoE2 transcription. In support of this hypothesis, we did not find an effect of high c-di-GMP levels on the transcription and activity of the SPI-1 TTSS transcriptional regulator HilA [143][149].

Induction of the pro-inflammatory cytokine IL-8 in HT-29 epithelial cells by *S. typhimurium*, is also inhibited by c-di-GMP signalling. Cellulose partially contributed to the inhibition of the IL-8 phenotype. The Römling group has shown previously, that in the absence of cellulose, enhanced binding of bacteria to epithelial cells can occur via curli fimbriae, which triggers an elevated immune response via curli-bound flagellin [27][92]. Moreover, CsgD is required for inhibition of IL-8 production from epithelial cells.

On the molecular level, secretion of monomeric flagellin was significantly inhibited by high c-di-GMP levels, while cell-associated flagellin was even enhanced, suggesting that non-inducible IL-8 phenotype of *S. typhimurium* with high c-di-GMP is due to a reduced amount of monomeric flagellin available to bind to TLR-5 and stimulate an im-

mune response. Deletion of *csgD* restored secretion of flagellin to wild type levels, which is sufficient to explain the stimulation of IL-8 production in this system. Consequently, CsgD interferes with both secretion of the SPI-1 TTSS effector protein SopE2 and monomeric flagellin. Flagellin is secreted to the tip of the flagellum through the TTSS flagellar apparatus for the assembly of flagellum [113][35]. Whether the same secretion pathway is responsible for the export of monomeric flagellin binding to TLR5 is not yet clear, but if so, CsgD expression interferes with the function of different type III secretion systems.

The cell-associated flagellin is attributed to the assembled flagellum and cannot be recognized by TLR-5 to induce IL-8 production [30]. Thus, when c-di-GMP is high, there might be more flagella; longer flagella, less easily shed or flagellin is more efficiently assembled into the flagellum [35]. In fact, stimulation of the secretion of monomeric flagellin has been observed in response to host cells [31] suggesting that secretion of monomeric flagellin occurs independently of the assembly of the flagellum. Flagellin secretion is a candidate target of c-di-GMP regulation under the environmental conditions investigated.

Overall, in Paper I we have shown that in *S. typhimurium*, under conditions that are otherwise favourable for virulence, c-di-GMP signalling in conjunction with the biofilm regulator CsgD and cellulose biosynthesis turns a highly invasive pathogen with immunostimmulatory properties into a non-invasive bacterium, which does not evoke an immune response. Indeed, c-di-GMP is considered to mediate the transition between acute and chronic infections [184][214]. In the nosocomial pathogen *P. aeruginosa* that establishes chronic infection of the lungs, c-di-GMP signaling is required for biofilm formation [189] and colonization. In *Vcholerae* low levels of c-di-GMP have been demonstrated to stimulate maximum expression of cholera toxin suggesting that c-di-GMP can either promote or inhibit virulence depending on the status of infection [197].

3.2 PAPER II

Contribution of GGDEF-EAL domain proteins to *Salmonella typhimurium* virulence phenotypes.

In Paper I, we have shown that at least two virulence properties, invasion and IL-8 production of HT-29 cells by *S. typhimurium* are regulated by c-di-GMP signaling. As c-di-GMP-affected phenotypes were tested by overexpression of a di-guanylate cyclase, which floaded the cell with c-di-GMP far over physiological levels, in paper II we investigated, which chromosomally encoded di-guanylate cyclases and

phosphodiesterases affect invasion and IL-8 induction. As virulence is a complex process, we also investigated the effect of the c-di-GMP signaling network on virulence by orally infecting streptomycin-treated mice, the mouse model of human gastroenteritis.

We used previously constructed single deletions of all identified GGDEF/EAL domain proteins that are demonstrated or putative di-guanylate cyclases or phosphodiesterases encoded by the *S. typhimurium* genome and tested for a phenotype in invasion and IL-8 production. GGDEF/EAL domain proteins are highly abundant in the genomes of most bacterial species suggesting that the c-di-GMP signalling network not only plays a fundamental role in bacterial signaling, but also that c-di-GMP signaling affects multiple physiological pathways, *samonella*). To recall, *S.typhimurium* possesses 20 GGGDEF/EAL domain proteins whereby 5 possess a GGDEF domain, 8 an EAL domain and 7 proteins consist of both, the GGDEF and EAL domain. Also other bacteria such as *Pseudomonas* and *V. cholerae* possess numerous GGDEF/EAL domain proteins suggesting a highly complex and fine tuned regulation of bacterial physiology by c-di-GMP signalling rather than an overall ON and OFF effect.

In Paper II, we demonstrate that distinct panels of individual GGDEF/EAL domain proteins specifically modulate *S. typhimurium* mediated invasion and IL-8 production of epithelial cells,*in vivo* colonization and systemic spread of the infection. Two distinct panels consisting of several GGDEF/EAL domain proteins contribute to each phenotype. Specifically, the mutants with deletions of the GGDEF domain proteins STM1283, STM1987 and STM4551, the EAL domain proteins STM0343, STM0468, STM1697, STM2215 ,STM3611 and STM4264 and the GGDEF-EAL domain protein STM2123 showed a statistically significant invasion phenotype, although the severity of the phenotype differed between the mutants. On the other hand, the mutants with deletions of the GGDEF domain protein STM1283, the EAL domain proteins STM0468 and STM4264 , the GGDEF-EAL domain STM1703 and STM2503 showed an IL-8 phenotype distinct from the wild type. These results show that distinct functions for individual GGDEF/EAL domain proteins in the virulence phenotypes. In addition, distinct panels of GGDEF/EAL domain proteins regulate the two virulence phenotypes under the same growth conditions. This type of regulation, of a phenotype by groups of GGDEF/EAL domain proteins, is consistent with the regulatory pattern seen previously. Rdar biofilm formation and motility are regulated by distinct panels of GGDEF/EAL domain proteins in *S. typhimurium* [192][232][217][241]. For example, at least

eight GGDEF/EAL domain proteins regulate the rdar morphotype of bacteria grown at 28°C on agar plates [192][232][217]. In *V. cholerae*, multiple GGDEF/EAL domains regulate the rugose phenotype of plate-grown cells [242][243].

For most of the mutants, we observed a phenotype consistent with the function of c-di-GMP signaling as elucidated in Paper I. Consequently, most GGDEF protein mutants showed an enhanced phenotype as compared to the wild type, while most EAL protein mutants showed a reduced phenotype. However, some phenotypes associated with mutated GGDEF/EAL domain proteins do not correlate with the predicted alterations in the level of this dinucleotide. For example, the mutant of the GGDEF domain protein STM1283 showed a reduced invasion rate, although STM1283 codes for a predicted di-guanylate cyclase. In comparison, the phenotype of the STM1283 mutant with respect to IL-8 production is consistent with STM1283 functioning as a di-guanylate cyclase.

In addition, it should be emphasized that the deletion of AdrA (STM0385) the highly active diguanylate cyclase overexpressed to create high intracellular concentrations of c-di-GMP under invasion conditions (Paper I) did not show any virulence phenotype AdrA might not be expressed from its natural promoter under invasion conditions, might not be active or simply might not affect virulence phenotypes, when expressed chromosomally. The phenotype of the individual EAL domain mutants could be complemented by YhjH, a stand-alone EAL domain protein with demonstrated c-di-GMP specific phosphodiesterase activity [177][244] This finding indicated, but did not entirely rigidly prove, that c-di-GMP specific phosphodiesterase activity, is responsible for the observed phenotypes of EAL mutants. The only exception was the STM0468 mutant. Neither its invasion nor its IL-8 induction phenotype could be complemented by YhjH, although bioinformatic analysis predicts a c-di-GMP specific phosphodiesterase activity for the EAL domain.

The presence of several c-di-GMP synthesizing and degrading proteins leads to the question whether proteins with the same enzymatic activity have a redundant function or work in different pathways. In Paper II, we addressed this question by the construction of double mutants of GGDEF or EAL domain proteins. In particular, we found that a double mutant of the EAL domain proteins STM3611 and STM4264 showed a significantly more reduction of invasion than the single mutants. Therefore, STM3611 and STM4264, the two c-di-GMP specific phosphodiesterases, which contribute the most to the invasion pheno-

type, have non-redundant functions. Most likely, those proteins also act in spatially and maybe even temporally distinct niches, to control distinct pathways, as it has been demonstrated before [192]. Further investigations will elucidate, which pathways are affected by STM3611 and STM4264.

Virulence is a complex process. We investigated the role of c-di-GMP signaling on the distinct virulence phenotypes invasion and IL-8 production *in vitro*, however, these phenotypes do not necessarily entirely correlate with the*in vivo* virulence as this was shown before in other pathogens [40][204]. We chose to study the role of c-di-GMP signaling in the streptomycin-treated mouse model [134] as this model reflects human gastroenteritis, the most common manifestation of infection of *S. typhimurium* in humans. Using competition experiments, we found that mutants of the GGDEF-EAL domain proteins STM2672, STM3615 and STM4551 were quickly outcompeted (latest at day 12) in the gastrointestinal tract. At day 34, additional mutants showed a milder colonization phenotype.

In conclusion, there is no overlap in the groups of mutants in GGDEF/EAL domain proteins that show a severe*in vivo* virulence phenotype and those that show *in vitro* phenotypes in invasion and IL-8 production. The long-term colonization phenotype is multifactorial and certainly includes more determinants than short-term (1h) invasion of epithelial cells.

In conclusion, in Paper II we could show that chromosomally encoded proteins involved in c-di-GMP metabolism, namely GGDEF/EAL domain proteins, contribute to*in vivo* and *in vitro* virulence of *S. typhimurium*. Distinct panels of GGDEF/EAL domain proteins influence different phenotypes showing the plasticity and flexibility of the c-di-GMP signaling network.

3.3 PAPER III

Impact of biofilm matrix components on interaction of commensal *Escherichia coli* with the gastrointestinal cell line HT-29.

Tight interaction of commensal bacteria such as *E. coli* with epithelial cells is considered to contribute to persistent colonization, but is also considered to be a virulence phenotype [245][246]. In contrast to *S. typhimurium*, in *E. coli* the biofilm components curli fimbriae and cellulose are expressed also at 37°C in vitro [247], suggesting that they could play a role in bacterial-host interactions. In Paper III, we investigated the effect of the expression of curli fimbriae and cellulose on the

interaction of commensal *E. coli* with the colon carcinoma cell line HT-29. In particular, we investigated adherence, invasion and induction of production of the pro-inflammatory cytokine IL-8. In addition to characterization of the role of curli fimbriae and cellulose to the above mentioned phenotypes, we found a distinct contribution of different flagellin H-serotypes to IL-8 induction.

Flagellation, motility and chemotaxis can be virulence factors in infections required for colonization and tissue invasiveness and the recruitment of host inflammatory cells [248][249][19]. On the other hand, expression of the flagella apparatus is turned off in the majority of cells of a commensal *E. coli* population upon intestinal colonization of mice [250].

In Paper III, we demonstrate that the biofilm matrix components curli fimbriae and cellulose expressed by the commensal *E. coli* strain TOB1 have a differential effect on interaction with the gastrointestinal epithelial cell line HT-29. While curli fimbriae promote adherence, internalization and IL-8 production, cellulose expression in the curli-expressing background inhibited these phenotypes. In other *E. coli* backgrounds, slightly different results were obtained. The Römling group found that cellulose is required for adhesion and enhanced cytokine production in the probiotic *E. coli* Nissle 1917 [251]. Others have found a synergistic effect of curli and cellulose on host cell adherence and biofilm formation of EHEC and EPEC [252]. In addition, we found a distinct role of different H serotypes, which reflect flagellin proteins with different sequences, in the interaction between commensal isolates of *E. coli* and HT-29 cells. Although we found in Paper III that expression of the biofilm matrix component curli fimbriae promotes IL-8 production in combination with flagellin, the highest IL-8 response was elucidated by *E. coli* Fec10, a strain which did not express curli fimbriae. Incubation of HT-29 cells with purified monomeric flagellin from strain Fec10 and TOB1 showed that flagellin from strain Fec10 was significantly more immunostimmulatory. In the HT-29 model system, IL-8 induction occurs since monomeric flagellin is recognized by TLR5. Residues in the N- and C-terminus of *S. typhimurium* flagellin FliC required for stimulation of IL-8 production by HT-29 cells were previously characterized [30]. Sequence comparison of the flagellins from strain Fec10 and TOB1 with flagellin from *S. typhimurium* showed that residues required for TLR5 recognition of flagellin showed a higher conservation in the flagellin of strain Fec10. This fact might explain the higher immunostimmulatory properties of Fec10 flagellin, which belongs to the H27 serotype group of flagellins.

3.4 PAPER IV

Characteristics of translocating *Escherichia coli* and the interleukin-8 response to infection.

Bacterial translocation is a virulence phenotype during bacterial overgrowth and in immunocompromised hosts [63]. In Paper IV, we characterized *E.coli* isolates from humans, pigs and rats which show a distinct translocation phenotype [253][254]. The *E.coli* strains were characterized with respect to biochemical phenotypes, serotypes and adherence and induction of a pro-inflammatory immune response in HT-29 cells. In addition, translocation from the apical to the baso-lateral side of an epithelial monolayer consisting of human-derived epithelial cells was investigated.

Translocating strains isolated from human and pig translocated most efficiently in this system. There was no correlation between the ability of a strain to adhere and to produce IL-8 and the ability to translocated. Previous studies have shown that internalization, but not adherence correlated with the translocation ability of an *E.coli* strain [63]. We observed, however, a significantly higher IL-8 induction in HT-29 by *E. coli* strain KIC-2. Purified flagellin of strain KIC-2 showed a similar significantly higher IL-8 induction.

The flagellin of strain KIC-2 is of serotype H21. The flagellin molecule can be divided into three regions [41], the conserved C1 at the N-terminus, the V variable region and the conserved C2 region at the C-terminus. Using the conserved N- and C-terminal regions, the H-serotypes of *E. coli* can be divided into two major groups EC1 and EC2. This division correlates also with the division of *E.coli* strains into different flagella morphotype groups [255]. Flagellins of the EC2 group are closely related to the FliC flagellin of S. enterica and their *fliC* gene may be derived from the *E. coli/Salmonella enterica* common ancestor. Members of the EC2 group comprise serotypes H2, H8, H11, H16, H21 and H27. Indeed, the two highly immunostimmulatory flagellins identified in Paper III and Paper IV, the F10 flagellin of serotype H27 and the KIC-2 flagellin of serotype H21, both belong to the EC2 group, suggesting that the EC2 group of flagellins has a highger immunostimmulatory capacity than the EC1 group. It would be worth to systematically investigate this hypothesis. In addition, the KIC-2 flagellin also showed higher conservation of the immunostimulatory TLR5 recognition amino acid signatures [30] than the flagellins from the EC1 group, which can explain the higher immunostimulatory effect.

In conclusion, in Paper III and IV, we have identified two *E.coli* flagellin serotypes, H21 and H27, which are more immunostimmulatory

than other investigated flagellin molecules. These two flagellin types belong to the EC2 subgroup of flagellins, which are closely related to the FliC flagellin of *S. typhimurium*. The FliC flagellin is highly immunostimmulatory with respect to the TLR5 mediated IL-8 response and the H21 and H27 flagellins seem to have retained these immunostimmulatory properties.

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